Diagenesis of the Organic Matrix in *Anadara trapezia* During the Late Quaternary: Preliminary Findings

MARGARET J. WHITELAW[1], BARRY D. BATTS[1], COLIN V. MURRAY-WALLACE[2] AND CHRISTOPHER R. MCRAE[1]

[1]Department of Chemistry, Macquarie University, North Ryde, NSW 2109; [2]School of Geosciences, University of Wollongong, Wollongong, NSW 2522

Whitelaw, M.J., Bats, B.D., Murray-Wallace, C.V. and McRae, C.R. (2001). Diagenesis of the organic matrix in *Anadara trapezia* during the Late Quaternary: Preliminary findings. *Proceedings of the Linnean Society of New South Wales* **123**, 225-234.

The concentration of amino acids found in the soluble organic matrix of modern and fossil shell of *Anadara trapezia* is quantified in this study. Results indicate that 91% of the total amino acids present in the soluble organic matrix are lost within 4,000 years, at an average rate of 3.25×10^{-3} pmol/µg per year. Over the next 2,000 years a further 6.3% are lost at an average rate of 4.49×10^{-4} pmol/µg per year and for a further 119,000 years 0.9% of material was lost at an average rate of 1.07×10^{-6} pmol/µg per year. Aspartic acid, glutamic acid, glycine and alanine were found to be present with the highest concentrations having a mean concentration of 28.3 pmol/µg. These amino acids were also found to be more readily hydrolyzed from the soluble organic matrix. After 4,000 years their concentrations had dropped to be within the same range as all amino acids remaining in the organic matrix with a mean concentration of 1.3 pmol/µg. Amino acid concentrations remained at this level for the next 119,000 years with little further losses.

The degree of racemization from L form to D form, i.e. the D/L ratio of amino acids was found to be related to concentration and amino acids such as aspartic acid, with high initial concentration also show faster racemization rates. Aspartic acid hydrolyses and racemizes at a faster rate than other amino acids leading to the hypothesis that the more aspartic acid molecules present in the protein strand, the greater the chance that aspartic acid will be in the optimum position for hydrolysis and racemization.

Manuscript received 14 February 2001, accepted for publication 22 August 2001.

KEYWORDS: mollusc, organic matrix, conchiolin, diagenesis, amino acid racemization, *Anadara trapezia*.

INTRODUCTION

Researchers from several scientific disciplines are studying the protein found in the shell matrix of molluscs. Material scientists have examined the way these organic matrix proteins, secreted by molluscs, regulate calcite and/or aragonite crystal formation within shells (Weiss et al. 2000). In particular, research has focused on biomineralization processes and their relevance to the synthesis of high performance nano-composite materials (Keith et al. 1992; Belcher et al. 1998). Geologists and archaeologists have used the degree of racemization of amino acids in shell and bone as a dating tool (Hare and Abelson 1968; Murray-Wallace 1993; Kimber et al. 1994; Harada and Handa 1995; Rutter and Blackwell 1995; Johnson and Miller 1997). Increasing amounts of D form

amino acids in fossil molluscan shell is an indication of "time-since-death". The rate of racemization is known to be genus dependent and these preliminary results are part of an ongoing study into the reasons for this dependency. All living organisms retain their amino acids in the L-form by enzyme action. When an organism dies, the enzyme suppression ceases and racemization reactions begin and will continue until equilibrium is attained. This process is highly sensitive to temperature and accordingly may have duration of 200 ka to 10 Ma (Miller and Brigham-Grette 1989). At equilibrium D/L equals 1 for enantiomers and approximately 1.3 for diastereoisomers (Miller and Brigham-Grette 1989; Murray-Wallace 1993). Stratigraphic correlation (Belperio et al. 1984; Murray-Wallace et al. 1999), phylogenetic studies (Degens and Spencer 1967; Robbins and Ostrom 1995), palaeotemperature studies (Cann and Clarke 1993; Miller et al. 1995) and the detection of reworking of fossils from older into younger sedimentary deposits (Murray-Wallace 1993; Rutter and Blackwell 1995; Wehmiller et al. 1995; Miller et al. 1997), represent other research applications of the racemization reaction of amino acids.

Knowledge of how the organic matrix breaks down over time is important when considering racemization of amino acids as a dating tool. It has long been established that temperature and the availability of water affect the rate of racemization in fossils, and that variations in the degree of racemization have been found between genera of the same age (Williams and Smith 1977; Lajoie et al. 1980; Wehmiller 1980; Rutter and Blackwell 1995; Roof 1997). The variation in the degree of racemization between genera of the same age is thought to be due to the presence of different proteins, characterized by more stable peptide bonds (Degens and Spencer 1967; Hare and Abelson 1968; Wehmiller 1984; Kaufman et al. 1992; Goodfriend et al. 1997). It has been established that amino acids at N-terminal positions of proteins tend to racemize fastest and that when the amino acid is in an internal position the rate of racemization is affected by the amino acids on either side (Mitterer and Kriausakul 1984; Mitterer 1993; Qian et al. 1995; Goodfriend 1997). The relative position of an amino acid in the protein chain will thus determine the rate of racemization.

This study examines the breakdown of the soluble organic matrix in the shell of one species of mollusc, the estuarine bivalve mollusc *Anadara trapezia*, over a period of 125,000 years. As diagenesis occurs, amino acids are hydrolyzed from the protein chain becoming free amino acids. In this study larger peptides and proteins have been extracted and amino acid concentrations have been determined. The concentration and composition of amino acids retained in protein and peptides over 12 kDa in size in fossil samples, is compared with the results for modern specimens. As routine studies have not quantified the concentrations of amino acids in fossil shell with any precision, this report is adding to knowledge in this field.

MATERIALS AND METHODS

Many current methods used to identify the amino acid composition of shell proteins involve demineralizing the shell using 6M HCl. The resultant solution is then analyzed for amino acid composition and concentration. The acid hydrolyses the intact proteins and peptide strands, as well as demineralizing the shell. Researchers then analyze the total acid hydrolysate and at times, free amino acids (Powell et al. 1989; Powell et al. 1991; Kaufman et al. 1992).

Analysis of total amino acid hydrolysate includes

- amino acids from the hydrolysis of the organic matrix,
- free amino acids from earlier diagenesis,
- free amino acids formed from the chemical breakdown of other amino acids, for example, Serine → Glycine + Alanine (Akiyama 1980), together with

- free amino acids that have diffused into the shell over time, that is, foreign organic material. This depends on the integrity of the shell matrix and the species concerned and studies have shown little in the way of non indigenous amino acids diffusing into shell (Miller and Hare 1980).

To overcome the problem of hydrolysis of protein associated with the use of 6M HCl, the method used in this study involves the gentle demineralization of the shelly material using a 10% solution of EDTA at pH 8 to remove Ca^{++}. The sample of ground shell is placed in a dialysis tube with a nominal pore size of 12 kDa allowing all free amino acids to diffuse through the tubing. Only intact proteins and larger peptide strands are retained (Wheeler et al. 1987; Halloran and Donachy 1995; Murray-Wallace et al. 2000).

Selected samples were chosen in an attempt to quantify the rate of change or loss of protein residues in shells during early diagenesis using one species, the bivalve *Anadara trapezia*. Modern shells were analyzed along with fossil samples of known age. Modern samples were collected live from Wallagoot Lake, near Eden, on the South Coast of New South Wales (Table 1). They were immediately frozen and then shucked prior to cleaning and grinding. Fossil samples of *Anadara trapezia* were collected from several geological coastal deposits in southern Australia (Table 1), where the age and origin of the deposits are well established (Murray-Wallace et al. 2000). Fossil shell samples were collected from sites, which today have similar mean annual air temperatures as it is likely that materials of the same age will have experienced similar diagenetic temperatures (Table 1).

Table 1.
Location of collection sites for modern and fossil samples used in this study and codes assigned to each fossil sample.

Age* (years)	Code	Location
0	Modern	Wallagoot Lake, South Coast, NSW
3,880 ± 60 (SUA-3059)	AWP	Wilson Memorial Park, Koona Bay, Lake Illawarra, NSW
6,280±60 (SUA-3058	Att	Tom Thumb Lagoon, Wollongong, NSW
6,800 ± 70 (SUA-3102)	Akb	Kully Bay, Lake Illawarra, NSW
125,000	Al	Largs, NSW
125,000	Awg	Watson's Gap, Chiton Rocks, SA

*Note: Conventional radiocarbon ages have been corrected for the marine reservoir effect for southern Australian ocean surface waters, (-450±35 years, Gillespie and Polach (1979)) and converted to sidereal years using the revised calibration program of Stuiver and Reimer (1993).

Shells for amino acid concentration analysis were cleaned by boiling 10 minutes in 30% H_2O_2 followed by boiling for 10 minutes in 50% bleach (2.6% sodium hypochlorite by weight). They were then washed in Milli-Q™ water and scrubbed lightly. Grinding of shells was achieved using a Retsch Muhle electric mortar and pestle. Finely powdered shell (20 g), was placed in Sigma 43 mm dialysis tubing with a nominal pore size of 12 kDa; 100 mL of 10% EDTA solution at pH 8 was added and the suspension placed in a 3 L conical flask with 1.5 L of the same EDTA solution. The flask was shaken in a Bioline orbital shaker at 180 rpm at 21° C, until demineralization was complete (about 3 days). The EDTA solution in the flask was completely replaced with fresh EDTA solution once during the process. The contents of the dialysis tubing were centrifuged at 20,000 ***g*** for 30 minutes at 10°C in a Sorvall RC-5B Superspeed centrifuge. The soluble matrix in the supernatant was decanted and the insoluble matrix frozen and stored for future investigation. The soluble matrix was then placed in dialysis tubing and washed exhaustively in Milli-Q™ water at 4°C in an electric shaker. The water was frequently changed to remove the EDTA. The content of the tubing was again centrifuged at 20,000 ***g*** at 10°C for 30 minutes and the supernatant liquid containing the soluble matrix was freeze-dried. After purification, 5mg/mL samples of the soluble matrix were acid hydrolyzed and analyzed for amino acid concentration and composition. Amino acid analysis was performed by the Australian Proteome Analysis Facility (APAF) using F-moc (9-Fluorenylmethyl chloroformate) chemistry, a sensitive method that allows detection of amino acids at low levels (down to 1 μg). The detection of F-moc derivatised amino acids is *via* fluorescence using the GBM Aminomate system.

Nitric acid and hydrochloric acid were trialed as demineralizing agents with the results showing that some hydrolysis of the shell protein occurred. EDTA, although a gentler reagent, has disadvantages. EDTA is difficult to remove completely from the final protein sample and interferes with the amino acid analysis. A blank sample was run and the results showed that peaks at the phenylalanine and tyrosine positions were produced and to a lesser extent at other amino acid wavelengths. This was overcome by deleting phenylalanine and tyrosine from the results and subtracting the blank (EDTA) values from the sample values for other amino acid peaks (Table 2).

Analysis for the extent of racemization followed the methods documented in Murray-Wallace (1993). Analysis of the N-pentafluoropropionyl-2 propyl esters was undertaken using a Hewlett-Packard 5850 Series II gas chromatograph fitted with a coiled, 25m capillary column with the stationary phase Chirasil-L-Val.

RESULTS

Protein was extracted from the shells, purified and analysed for amino acid concentration and composition. Analysis showed that the loss of amino acids was rapid over the first 4,000 years from the onset of racemization, and maintained a relatively stable concentration from 4,000 years to 125,000 years (Fig.1). Over the first 4,000 years 91% of original material was lost at an average rate of 3.25×10^{-3} pmol/μg per year. Over the next 2,000 years a further 6.3% was lost at an average rate of 4.49×10^{-4} pmol/μg per year. Over the final 119,000 years, 0.9% of material was lost at an average rate of 1.07×10^{-6} pmol/μg per year. The curve for the average depletion rate of amino acids is shown in Figure 3 and has the equation $y = 12.221x^{-2.8517}$ with an R^2 of 0.9783.

In the modern shells three groups of amino acids are apparent based on their relative concentrations (Fig. 1). A group with high concentrations (mean of 28.3 pmol/μg), a group with moderate concentrations (mean of 15.7 pmol/μg) and a third group with quite low concentration level (mean of 1.4 pmol/μg). After 4,000 years the low-level amino acids have completely disappeared and therefore disregarded in this study,

Table 2. Concentrations are shown for the six shell samples. The first column shows the concentration in pmol per 75µg sample. The second column shows the apparent concentration obtained for each amino acid when a blank sample of EDTA was run and column three is the adjusted concentration, in pmol/µg obtained by subtracting the blank values from the sample values.

Modern	Sample	Blank	Pmol/ug	**AWP**	Sample	Blank	Pmol/ug	**Att**	Sample	Blank	Pmol/ug
Asp	2775.26	2.93	36.96	Asp	127.19	2.93	1.66	Asp	19.8	2.93	0.22
Glu	2021.45	2.64	26.92	Glu	148.17	2.64	1.94	Glu	23.11	2.64	0.27
Ser	1145.26	3.52	15.22	Ser	160.29	3.52	2.09	Ser	17.24	3.52	0.18
Gly	1925.79	16.31	25.46	Gly	374.28	16.31	4.77	Gly	161.65	16.31	1.94
Arg	537.46	0.73	7.16	Arg	81.31	0.73	1.07	Arg	20.49	0.73	0.26
Thr	953.99	1.56	12.70	Thr	85.88	1.56	1.12	Thr	15.81	1.56	0.19
Ala	1782.83	1.51	23.75	Ala	93.94	1.51	1.23	Ala	30.32	1.51	0.38
Pro	2391.83	2.88	31.85	Pro	91.49	2.88	1.18	Pro	424.35	2.88	5.62
Tyr	8.16	1.29	0.09	Tyr	26.56	1.29	0.34	Tyr	17.71	1.29	0.22
Val	773.41	0.14	10.31	Val	83.05	0.14	1.11	Val	60.91	0.14	0.81
Ile	576.55	11.85	7.53	Ile	68.56	11.85	0.76	Ile	56.48	11.85	0.60
Leu	741.04	2.45	9.85	Leu	85.14	2.45	1.10	Leu	46.64	2.45	0.59
Lys	572.15	1.76	7.61	Lys	39.66	1.76	0.51	Lys	18.52	1.76	0.22

Awg	Sample	Blank	pmol/ug	**Al**	Sample	Blank	pmol/ug	**Akb**	Sample	Blank	pmol/ug
Asp	14.31	2.93	0.15	Asp	26.02	2.93	0.31	Asp	78.1	2.93	1.00
Glu	9.8	2.64	0.10	Glu	21.91	2.64	0.26	Glu	68.96	2.64	0.88
Ser	10.13	3.52	0.09	Ser	22.34	3.52	0.25	Ser	82.14	3.52	1.05
Gly	74.34	16.31	0.77	Gly	133.23	16.31	1.56	Gly	401.38	16.31	5.13
Arg	5.57	0.73	0.06	Arg	20.46	0.73	0.26	Arg	113.71	0.73	1.51
Thr	7.71	1.56	0.08	Thr	18.87	1.56	0.23	Thr	110.9	1.56	1.46
Ala	11.98	1.51	0.14	Ala	24.2	1.51	0.30	Ala	94.77	1.51	1.24
Pro	102.87	2.88	1.33	Pro	232.89	2.88	3.07	Pro	756.52	2.88	10.05
Tyr	134.16	1.29	1.77	Tyr	269.91	1.29	3.58	Tyr	78.72	1.29	1.03
Val	40.56	0.14	0.54	Val	66.34	0.14	0.88	Val	184.85	0.14	2.46
Ile	21.61	11.85	0.13	Ile	66.12	11.85	0.72	Ile	210.04	11.85	2.64
Leu	19.63	2.45	0.23	Leu	55.51	2.45	0.71	Leu	197.05	2.45	2.59
Lys	9.41	1.76	0.10	Lys	14.29	1.76	0.17	Lys	61.55	1.76	0.80

Figure 1. Depletion of amino acids with time. Aspartic acid shows a higher concentration in fossil samples relative to the other amino acids. There are three groups of amino acids, Group A with high initial concentrations, Group B with moderate initial concentrations and Group C with low initial concentrations. Amino acids are listed from top to bottom on the figure. It is to be noted that there are three amino acids with very similar concentration, isoleucine, arginine and lysine, seen as a single dark line in Group B.

Group A	Group B		Group C
Aspartic acid		**Serine**	**Methionine**
Glutamic acid		Threonine	Histidine
Glycine	Valine		
Alanine	Leucine		
		Lysine	
		Isoleucine	
		Arginine	

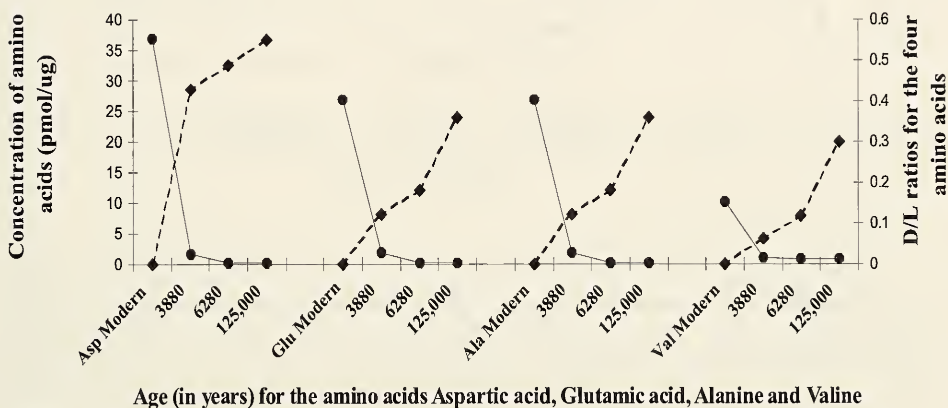
Table 3. Concentrations of four amino acids and the corresponding D/L ratios for those amino acids from modern and fossil samples

Age	Amino acid concentration (pmol/µg)				Amino acid D/L ratio (years) (in fossil samples)			
	Asp	Glu	Ala	Val	Asp	Glu	Ala	Val
0	39.96	26.92	23.75	10.31	-	-	-	-
3,880	1.66	1.94	1.23	1.11	0.42	0.12	0.22	0.06
6,280	0.22	0.27	0.38	0.81	0.49	0.18	0.32	0.12
6,800	1.00	0.88	1.24	2.46	NA	0.27	0.30	0.12
125,000	0.31	0.26	0.30	0.88	0.55	0.36	0.60	0.30

but for all the other amino acids, after 4,000 years, their concentrations are within the same range, (mean of 1.34 pmol/μg) and although low, are still detectable. Amino acid concentrations remain at this level for the next 120,000 years with very little further losses.

D/L ratios are reported for the enantiomeric amino acids alanine, valine, glutamic acid and aspartic acid (Table 3). D/L ratios for the four amino acids are reported for all fossil samples. The ratios show an interesting relationship with the original concentration of each amino acid in modern shell. As expected, with each of the four amino acids, the D/L ratio increases with time as amino acids racemize and approach equilibrium. The concentration of amino acids in the unhydrolyzed organic matrix is at the same time decreasing (Fig. 2).

Figure 2. Composite graph which illustrates the depletion of four amino acids from the organic matrix, (shown as hatched lines) and the corresponding increase in D/L ratio of those amino acids, with time.

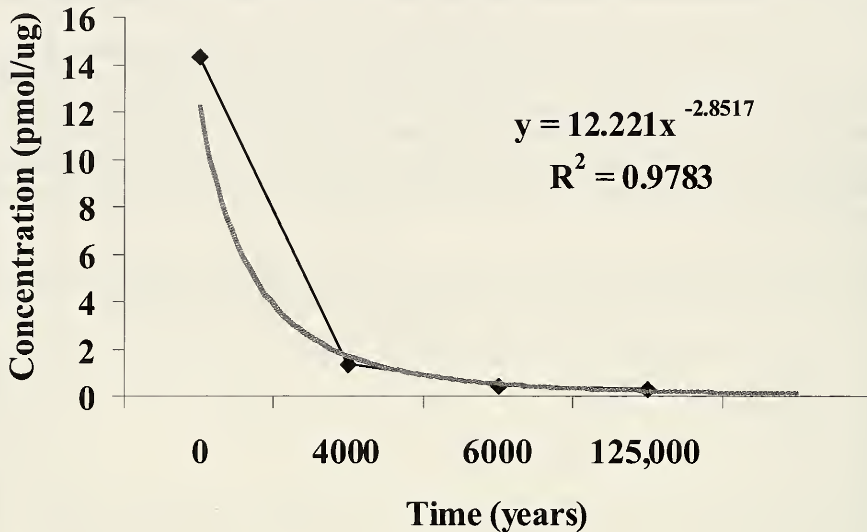


There is a correlation between the D/L ratio and the original concentration of a given amino acid in the modern shell. Aspartic acid was originally present in *Anadara trapezia* with the highest concentration. After some 4,000 years since the onset of racemization, the concentration dropped to levels comparable to most other amino acids in the sample and yet the aspartic acid D/L ratios in fossil samples tend to be higher than for the other amino acids (Fig. 3). The high concentration of aspartic acid found in the modern organic matrix suggests that there is a greater chance that an aspartic acid residue will be in the terminal position of the protein and hence in a position where racemization may readily occur. Hence we hypothesize that the higher the concentration of an amino acid the greater the chances of its occurrence at terminal positions during diagenesis and hence the more likely that racemization will occur thus accounting for the higher rate of racemization experienced by that acid (in this case, aspartic acid). This hypothesis is in agreement with the finding that acidic amino acid residues are found in every biomineralized system examined to date (Weiner et. al 1983).

DISCUSSION

The concentration of aspartic acid falls dramatically over 4,000 years possibly indicating that aspartic acid is hydrolyzing rapidly from the protein and forming free amino acids. These free amino acids are undergoing racemization over this period of time.

Figure 3. Average depletion curve. When the average of all the amino acid concentrations is plotted against time, it is possible to plot a power curve that fits the data very well with an $R^2 > 0.97$. The equation of the curve is $y=12.2x^{-2.85}$ where x represents time and y is concentration.



One sample, Akb, dated at 6,800± 70 years, was found to have higher concentrations for all amino acids when compared with another sample Att, of similar age, (6,280±60 years), and geological setting. Both samples related to the same set of geological processes: quiet water, coastal lagoon which formed after the sea level rose to its present position 7,000 years ago (Young et al. 1993). It is speculated that the preservation of shells collected in Kully Bay, Lake Illawarra (Akb) was better than conditions experienced at Tom Thumb Lagoon, Wollongong (Att) thus retarding the hydrolysis of the conchiolin proteins. This could be due to differing local environmental conditions or individual variations in the sample shells. The extent and effect of boring animals in shell samples could account for discrepancies, as would other natural variations. An alternate interpretation for the discrepancy would be that whilst in situ, low molecular weight peptides and free amino acids were preferentially lost from perhaps only one shell in the sample through in situ leaching. As the amino acid concentrations are derived using several shell samples and the D/L ratios are calculated for the total amino acid hydrolysate, the loss of lower molecular weight peptides and free amino acids could produce this discrepancy. The results for Akb are shown in Table 2 but for clarity are not included when comparing amino acid depletion with time.

CONCLUSIONS

1 Hydrolysis of the shell protein is initially rapid with over 90% of original material lost within 4,000 years, at an average rate of 3.25×10^{-3} pmol/µg per year. The remaining protein is relatively stable and this study shows that over the next 120,000 years only 0.9% of material was lost at an average rate of 1.07×10^{-6} pmol/µg per year.

2 Regardless of whether amino acids are present in high or moderate concentration in the protein of modern shell, their concentrations fall to approximately the same level after 4,000 years, (with a mean of 1.34 pmol/ μg), from the onset of diagenesis. As hydrolysis proceeds and the protein breaks down into peptide strands, amino acids will increasingly be found in optimum positions for racemization and the D/L ratio for each amino acid will increase. Graphs of D/L ratios of individual amino acids versus their concentration in unhydrolysed organic matrix show these expected trends.

3 If an amino acid is present in high concentrations in modern shell it will also show a higher rate of racemization in fossil samples. The rate of increase of the D/L ratio for aspartic acid was found to be much greater than that of the other amino acids considered in this study. As there is a higher concentration of aspartic acid molecules in the peptide strand, we suggest that there is a greater chance that aspartic acid will be found at the optimum N-terminal during hydrolysis and so racemize faster.

REFERENCES

Akiyama, M. (1980). Diagenetic decomposition of peptide-linked serine residues in fossil scallop shells. In "Characterization of proteins,peptides and amino acids". Quaternary Biogeochemistry of Amino Acids. Conference Papers (Eds P.E. Hare, T.C. Hoering and K. King) pp. 115-120. (Wiley: New York).

Belcher, A.M., Hansma, P.K., Stucky, G.D. and Morse, D.E. (1998). First steps in harnessing the potential of biomineralization as a route to new high-performance composite materials."*Acta Mater* **46**, 733-736.

Belperio, A.P., Smith, B.W., Polach, H.A., Nittrouer, C.A., DeMaster, D.J., Prescott, J.R., Hails, J.R. and Gostin, V.A. (1984). Chronological studies of the Quaternary marine sediments of northern Spencer Gulf, South Australia. *Marine Geology* **61**, 265-296.

Cann, J.H. and Clarke, J.D.A. (1993). The significance of *Marginopora vertebralis* (Foraminifera) in surficial sediments at Esperance, Western Australia, and in last interglacial sediments in northern Spencer Gulf, South Australia. *Marine Geology* **111**, 171-187.

Degens, E.T. and Spencer, D.W. (1967). Palaeobiochemistry of molluscan shell proteins. *Comparative Biochemical Physiology B.* **20**, 553-579.

Gillespie, R. and Polach, H.A. (1979). The suitability of marine shells for radiocarbon dating of Australian prehistory. In 'Proceedings Ninth International Conference on Radiocarbon Dating'. (Eds R. Berger and H. Suess) pp. 404-421. (University of California Press: California).

Goodfriend, G. Flessa, K.W. and Hare, P.E. (1997). Variation in amino acid composition among shell layers in the bivalve *Chione* from the Gulf of California. *Geochimica et Cosmochimica Acta.* **61**, 1487-1493.

Halloran, A.B. and Donachy, J.E. (1995). Characterization of organic matrix macromolecules from the shells of the Antarctic scallop, *Adamussium colbecki. Comparative Biochemistry Physiology B.* **2**, 221-231.

Harada, N. and Handa, N. (1995). Amino acid chronology in the fossil planktonic foraminifera, *Pulleniatina obliquiloculata* from the Pacific Ocean. *Geophysical Research Letters.* **22**, 2353-2356.

Hare, P.E. and Abelson, P.B. (1968): Racemization of amino acids in fossil shells. In 'Carnegie Institute of Washington Yearbook'. Vol. 66. pp. 526-528.

Johnson, B.J. and Miller, G.H. (1997). Archaeological applications of amino acid racemization. *Archaeometry.* **38**, 265-287.

Kaufman, D.S., Miller, G.H. and Andrews, J.T. (1992). Amino acid composition as a taxonomic tool for molluscan fossils: an example from Pliocene-Pleistocene Arctic marine deposits. *Geochimica et Cosmochimica Acta.* **56**, 2445-2453.

Keith, J.A., Stockwell, S.A., Ball, D.H., Muller, W.S., Kaplan, D.L., Thannhauser, T.W. and Sherwood, R.W. (1992). Characterization of the complex matrix of the *Mytilus edulis* shell and the implications for biomimetic ceramics. *Hierachically structured materials. Proceedings Materials Research Society.* **255**, 3-8.

Kimber, R.W.L., Kennedy, N.M. and Milnes, A.R. (1994). Amino acid racemization dating of a 140 000 year old tephra-loess-paleosol sequence on the Mamaku Plateau near Rotorua, New Zealand. *Australian Journal of Earth Sciences.* **41**, 19-26.

Lajoie, K.R., Wehmiller, J.F. and Kennedy, G.L. (1980). Inter- and intrageneric trends in apparent racemization kinetics of amino acids. In 'Quaternary mollusks. Biogeochemistry of amino acids. Conference Papers'. (Eds P.E. Hare, T.C. Hoering and K.King Jr.). pp. 305-339 (Wiley: New York).

Miller, G.H. and Brigham-Grette, J. (1989). Amino acid geochronology: resolution and precision in carbonate fossils. *Quaternary International* .**1**, 111-128.

Miller, G.H. and Hare, P.E. (1980). Amino acid geochronology: Integrity of the carbonate matrix and potential of molluscan fossils. In 'Biogeochemistry of Amino Acids'. (Eds P.E. Hare, T.C. Hoering and K. King). pp. 415-451. (Wiley: New York).

Miller, G.H., Magee, J.W. and Jull, A.J.T. (1997). Low-latitude glacial cooling in the Southern Hemisphere from amino-acid racemization in emu eggshells. *Nature*. **385**, 241-244.

Mitterer, R.M. (1993). The diagenesis of proteins and amino acids in fossil shells. 'Organic Geochemistry' (Eds M.H. Engel and S.A. Macko). pp. 739-753. (Plenum Press: New York).

Mitterer, R.M. and Kriausakul, N. (1984). Comparison of rates and degrees of isoleucine epimerization in dipeptides and tripeptides. *Organic Geochemistry*. **7**, 91-98.

Murray-Wallace, C.V. (1993). A review of the application of the amino acid racemization reaction to archaeological dating. *The Artifact*. **16**, 19-26.

Murray-Wallace, C.V., Belperio, A.P., Bourman, R.P., Cann, J.H. and Price, D.M. (1999). Facies architecture of a last interglacial barrier: a model for Quaternary barrier development from the Coorong to Mount Gambier coastal plain, southeastern Australia. *Marine Geology* .**158**, 177-195.

Murray-Wallace, C.V., Beu, A.G., Kendrick, G.W., Brown, L.J., Belperio, A.P. and Sherwood, J.E. (2000). Palaeoclimatic implications of the occurrence of the arcoid bivalve *Anadara trapezia* (Deshayes) in the Quaternary of Australasia. *Quaternary Science Reviews*. **19**, 559-590.

Powell, E.N., King, J.A. and Boyles, S. (1991). Dating time-since-death of oyster shells by the rate of decomposition of the organic matrix. *Archaeometry* **33**, 55-68.

Powell, E.N., Logan, A., Stanton Jr., R.J. and Davies, D.J. (1989). Estimating time-since-death from the free amino acid content of the mollusc shell: a measure of time averaging in modern death assemblages? Description of the technique. *Palaios*. **4**, 16-31.

Qian, Y., Engel, M.H., Goodfriend, G.A. and Macko, S.A. (1995). Abundance and stable isotope composition of amino acids in molecular weight fractions of fossil and artificially aged mollusk shells. *Geochimica et Cosmochimica Acta*. **59**, 1113-1124.

Robbins, L.L. and Ostrom, P.H. (1995). Molecular isotopic and biochemical evidence of the origin and diagenesis of shell organic material. *Geology*. **23**, 345-348.

Roof, S. (1997). Comparison of isoleucine epimerisation and leaching potential in the molluskan genera *Astarte*, *Macoma*, and *Mya*. *Geochimica et Cosmochimica Acta*. **61**, 5325-5333.

Rusenko, K.W., Donachy, J.E. and Wheeler, A.P. (1991). Purification and characterization of a shell matrix phosphoprotein from the American oyster. In 'Surface reactive peptides and polymers'. (Ed K.W. Rusenko) pp. 107-124. (American Chemical Society).

Rutter, N.W. and Blackwell, B. (1995). Amino acid racemization dating. In 'Dating Methods for Quaternary deposits'. (Eds N.W. Rutter and N.R. Catto) pp. 125-164. (Geological Association of Canada, Geotext2).

Stuiver, M. and Reimer, P.J. (1993). Extended ^{14}C data base and revised calibration 3.0 ^{14}C age calibration program. *Radiocarbon*. **35**, 215-230.

Wehmiller, J.F. (1980). Intergenetic differences in apparent racemization kinetics in mollusks and foraminifera: implications for models of diagenetic racemization. In 'Biogeochemistry of amino acids'. (Eds P.E. Hare, T.C. Hoering and K. King Jr.) pp. 341-355. (Wiley: New York).

Wehmiller, J.F. (1984). Relative and absolute dating of Quaternary molluscs with amino acid racemization: evaluation, applications and questions. In 'Quaternary dating methods'. (Ed W.C. Mahaney) pp. 171-193. (Elsevier Science:Amsterdam).

Wehmiller, J.F., York, L.L. and Bart, M.L. (1995). Amino acid racemization geochronology of reworked Quaternary mollusks on U.S. Atlantic coast beaches: implications for chronostratigraphy, taphonomy and coastal sediment transport. *Marine Geology*. **124**, 303-337.

Weiner, S., Traub, W. and Lowenstam, H.A. (1983). Organic matrix in calcified exoskeletons. In 'Biomineralization and biological metal accumulation'. (Ed. P. Westbrock and E.W. de Jong), pp. 205-224. (Dordrecht, Netherlands: Reidel).

Weiss, I.M., Kaufmann, S., Mann, K. and Fritz, M. (2000). Purification and characterization of Perlucin and Perlustrin, two new proteins from the shell of the mollusc'*Haliotis laevigata*. *Biochemical and Biophysical Research Communications* . **267**, 17-21.

Wheeler, A.P., Rusenko, J.W., George, J.W. and Sikes, C.S. (1987). Evaluation of calcium binding by molluscan shell organic matrix and its relevance to biomineralization. *Comparative Biochemistry and Physiology*. **87B**, 953-960.

Williams, K.M. and Smith, G.G. (1977). A critical evaluation of amino acid racemization to geochronology and geothermometry. *Origins of Life*. **8**, 91-144.

Young, R.W., Bryant, E.A., Price, D.M., Wirth, L.M. and Pease, M (1993).Theoretical constraints and chronological evidence of Holocene coastal development in central and southern New South Wales, *Australian Geomorphology*.**7**, 317-329.